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HOW Is Required for Stem Cell Maintenance in the *Drosophila* Testis and for the Onset of Transit-Amplifying Divisions

Adrian C. Monk,[1,2] Nicole A. Siddall,[1,2] Talila Volk,[3] Barbara Fraser,[2,4] Leonie M. Quinn,[1] Eileen A. McLaughlin,[2,4] and Gary R. Hime[1,2,*]
[1]Department of Anatomy and Cell Biology, University of Melbourne, Parkville, Victoria 3010, Australia
[2]ARC Centre of Excellence in Biotechnology and Development, Callaghan, NSW 2308, Australia
[3]Department of Molecular Genetics, Weizmann Institute, Reohovat 76100, Israel
[4]School of Environmental & Life Sciences, University of Newcastle, Callaghan, NSW 2308, Australia
*Correspondence: g.hime@unimelb.edu.au
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SUMMARY

The mechanisms by which germline stem cells (GSCs) in the *Drosophila* testis undergo asymmetric division to regenerate a stem cell as well as a daughter (gonialblast) that will only undergo a further four mitotic divisions prior to entering premeiotic S phase and differentiating into a cyst of spermatocytes are not fully resolved. Here we demonstrate that the HOW RNA-binding protein is required for maintenance of CycB and therefore mitotic progression in GSCs and gonialblasts as well as determining the timing of the spermatogonial divisions. HOW is normally expressed in a complementary pattern to Bam in the germline and *bam* mRNA is bound by HOW in vivo. Ectopic expression of the HOW(L) isoform is associated with a delay in accumulation of Bam to the level required for differentiation, resulting in extra mitotic divisions. Spatiotemporal regulation of HOW expression is therefore required to specify the four spermatogonial transit-amplifying divisions.

INTRODUCTION

The regulation of stem cell proliferation and differentiation is a tightly controlled process that ensures tissue homeostasis without either losing the stem cell pool or producing a neoplasm of undifferentiated cells. The number of differentiated progeny produced per stem cell division is dependent upon the number of post-stem cell "transit-amplifying" (TA) divisions. The number of TA divisions is plastic in some tissues and can respond to extracellular signals (e.g., during vertebrate hematopoiesis) (reviewed in Krause, 2002) but appears to be an invariant stereotypical number in other tissues (e.g., the *Drosophila* testis) (Fuller, 1993). Little is currently understood about the mechanisms that set the number of TA divisions in most tissues.

The male and female GSC niches of *Drosophila* have been used extensively to uncover developmental regulators of stem cell behavior (Fuller, 1993; Xie et al., 2005). The well-defined cellular architecture of the stem cell niche has enabled effects of genetic alterations to be easily observed at the level of a single cell. The male GSC niche consists of a group of somatic hub cells that anchor 8–10 GSCs in a rosette arrangement around the hub (Figure 1A). Each GSC is also surrounded by a pair of somatic stem cells (SSCs) or cyst progenitor cells. The SSCs receive a short-range signal from the hub via a secreted cytokine-like molecule, Unpaired (Upd). Upd binds to the Domeless receptor on SSCs thereby activating the *Drosophila* JAK/STAT signaling pathway, which subsequently communicates to GSCs thereby promoting GSC maintenance (Leatherman and Dinardo, 2008). Concomitant with division of a GSC, the associated SSCs also undergo division such that the resultant GSC daughter committed to differentiation (the gonialblast) is surrounded by a pair of somatic cyst cells that no longer divide but continue to regulate germ cell division. The gonialblast undergoes a stereotypical four more rounds of spermatogonial (TA) mitoses, characterized by incomplete cytokinesis, prior to differentiating into a spermatocyte (Fuller, 1993). The cyst cells are essential for regulating the number of TA divisions as disruption to signaling between the cyst cells and the germ cells results in aberrant spermatogonial division (Kiger et al., 2000; Tran et al., 2000).

A number of other signaling pathways have been implicated in regulating GSC maintenance and differentiation. The two BMP-like molecules, Dpp and Gbb, are secreted by somatic components of the testis and prevent GSC differentiation by inhibiting expression of the differentiation factor Bam (Kawase et al., 2004; Shivdasani and Ingham, 2003). Recent work has shown that the switch from TA divisions to meiotic differentiation is triggered by accumulation of Bam to a critical level in combination with the rate of cell cycle within the accumulation period (Insco et al., 2009).

Recent work in vertebrates has revealed a major role for negative regulators of gene expression in stem cell maintenance. This may be due to the requirement for a large range of differentiation factors to be kept silent in stem cells in order to maintain their pluripotent state (Boyer et al., 2006; Lee et al., 2006). In line with these observations, *Drosophila* male GSCs also appear to require function of repressors of gene activation in order to maintain a stem cell fate. Previously, we showed that the translational repressor, Musashi, is required in GSCs to prevent them from prematurely differentiating into TA spermatogonia (Siddall et al., 2006). Here we describe the role of the developmental

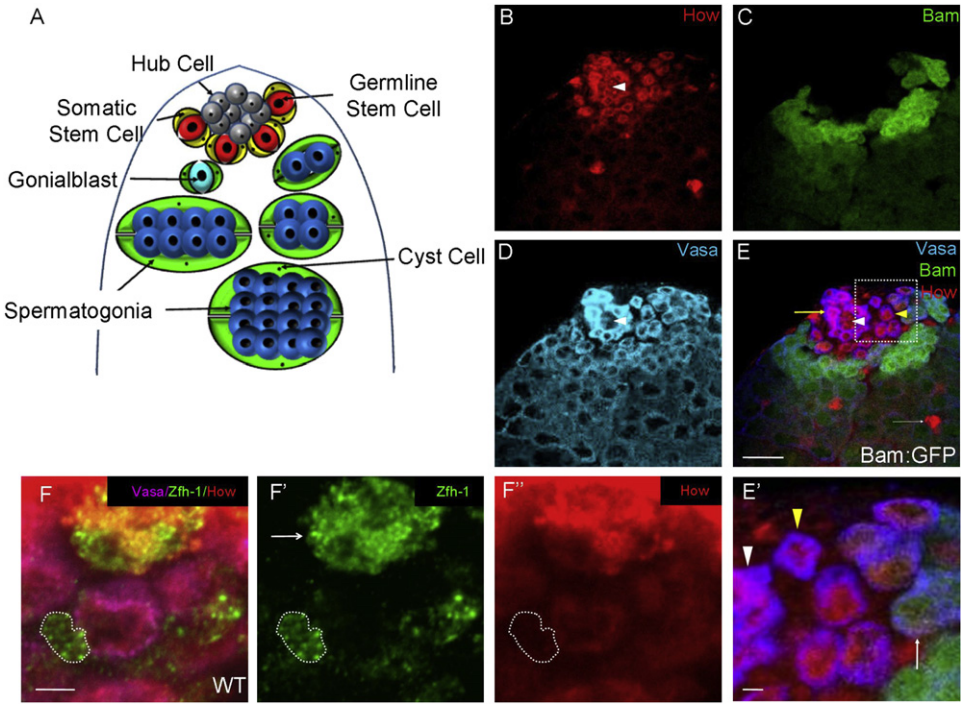


Figure 1. *how* Is Expressed in Early Germ Cells

(A) Schematic of the testis apex. GSCs (red) and SSCs (yellow) are anchored to somatic hub cells (gray). A GSC divides asymmetrically to produce a gonialblast (light blue), which produces 16 spermatogonia (dark blue) and is encapsulated by two somatic cyst cells (green).

(B–E) Anti-HOW labels GSCs (yellow arrow) and gonialblasts (yellow arrowhead) as well as 2-cell spermatogonia. HOW levels are reduced by the 4-cell stage when Bam expression is first detected (*bam::*GFP reporter). HOW is present in hub cells (white arrowhead) and cyst cell nuclei (white arrow).

(E′) High magnification of the box in (E), showing a GSC (white arrowhead), a gonialblast (yellow arrowhead), and a spermatogonium at the 4-cell stage (white-arrow).

(F–F″) Anti-Zfh-1 (green) marks hub cells (white arrow) and SSCs (white dotted line). HOW is detected in GSCs and hub cells, but not in SSCs.

Scale bars represent 20 μm in (A)–(E); 5 μm in (E′) and (F). See also Figure S1.

regulator, Held-out-wings (HOW), in GSC maintenance and germ cell division.

HOW is an RNA-binding protein that was originally identified from a hypomorphic mutation that produced adult flies with a wing posture defect (Baehrecke, 1997). Analysis of null alleles of *how* revealed a requirement for HOW function in differentiation of embryonic muscle tendon cells (Nabel-Rosen et al., 1999). Tendon cell differentiation is regulated by the relative levels of two HOW splice isoforms that generate proteins with different properties. In tendon precursor cells, HOW(L) binds to the mRNA encoding the key modulator of tendon cell differentiation, Stripe, leading to its degradation. HOW(S) lacks the C-terminal nuclear retention domain and hence it permits export of the *stripe* mRNA, thus stabilizing the mRNA and enabling Stripe protein accumulation and tendon cell differentiation (Nabel-Rosen et al., 1999, 2002).

This mechanism of HOW function appears to be evolutionarily conserved as indicated by the fact that HOW has orthologs in *C. elegans* and vertebrates, GLD-1 and QKI, respectively (reviewed in Volk et al., 2008). It is as yet unknown how many target genes are regulated by HOW or indeed how many tissue types require HOW function for correct differentiation. During embryonic mesoderm formation, HOW downregulates levels of the Cdc25 homolog Stg, which is essential and rate limiting for mitosis (Edgar and Datar, 1996). Stg triggers mitotic entry by dephosphorylating, and thereby activating the Cdk1/CycB kinase (Edgar and Datar, 1996). HOW, therefore, prevents mitotic progression during invagination of the presumptive mesoderm by behaving as a repressor of *stg* expression (Nabel-Rosen et al., 2005). Later in embryonic development, HOW regulates mesoderm spreading via suppression of an alternate target gene, *miple1,* which subsequently leads to activation of MAP kinase signaling (Toledano-Katchalski et al., 2007).

Thus a number of tissue types require HOW function for differentiation and HOW regulates multiple targets, which suggests we are far from knowing the full complement of HOW target genes. In this study we define a role for HOW in regulation of spermatogonial division. The absence of HOW results in loss of GSCs and spermatogonial arrest in G2 phase of the cell cycle, which is associated with decreased levels of CycB. These findings suggest that HOW is required to maintain levels of CycB in GSCs and spermatogonia. In addition, downregulation of HOW suppresses the delay in differentiation observed in *bam* heterozygotes. Ectopic expression of HOW(L) delays accumulation of Bam in spermatogonia, thereby allowing further mitotic divisions prior to differentiation. The normal domain of HOW expression is complementary to that of Bam and we found that HOW binds to *bam* mRNA in vivo. Thus, HOW is

needed both to maintain GSCs and for an early phase of cyst formation.

RESULTS

how Is Specifically Expressed in Early Germ Cells in the *Drosophila* Testis

To examine the expression pattern of *how*, we used a specific polyclonal antibody (Nabel-Rosen et al., 1999) and immunostained testes carrying a *bam::GFP* transgene (Chen and McKearin, 2003). Testes from this transgenic line appear phenotypically wild-type and express *bam*-driven GFP from the 4-cell spermatogonial stage to the 16-cell stage (Figure 1C). HOW protein was detected in Vasa-expressing GSCs, their immediate daughter cells (gonialblasts), and 2-cell spermatogonial cysts (Figures 1B and 1E). HOW expression was downregulated at the 4-cell stage, the time *bam::GFP* expression is normally first observed (Figure 1E). Additionally, HOW protein was detected in somatic hub cells and the nuclei of mature cyst cells (Figure 1E); however, HOW protein was not detected in somatic stem cells (SSCs) (Figures 1F–1F″) and genetic analyses suggest that HOW does not function in SSCs (Figure S1 available online). The pattern of HOW expression is unique among genes expressed in the *Drosophila* male germline and suggests that HOW may have a function confined to very early germ cells, including the GSCs.

HOW Is Required for GSC Maintenance

To determine whether HOW is required for *Drosophila* GSC division, maintenance, or differentiation, we examined adult testes from several different *how* loss-of-function (LOF) flies. Because *how* null mutants are embryonic lethal (Baehrecke, 1997), we analyzed transheterozygotes for the how^{r17} hypomorphic and how^{r4} complete LOF alleles, which survive to the late pupal stage (Baehrecke, 1997). In WT testes, a ring of tightly compacted Vasa-expressing cells can be identified as GSCs because of their position surrounding the hub (Figure 2A). In contrast, $how^{r17/r4}$ hypomorphs display a loss of Vasa-positive germ cells abutting the hub (Figure 2B), with 36% of testes analyzed completely lacking GSCs (n = 25). Another 16% of testes showed significant gaps in the ring of Vasa-positive cells adjacent to the hub, suggesting that GSCs are preferentially lost in *how* mutants, ultimately resulting in a complete loss of the germline. Furthermore, remaining *how* mutant germ cells were not found in organized cysts and different-sized germ cells were observed near the hub (Figure 2B). Although a somatic hub was observed in the hypomorphic combination (Figure 2B), the germline phenotype could reflect a requirement for HOW function in germ cells, somatic cells, or both cell populations. Therefore, we obtained a *UAS:*how^{RNAi} strain (VDRC) (Dietzl et al., 2007) to knock down the levels of all HOW isoforms in specific cell types via the binary Gal4 UAS system. We drove expression of the shRNA transgene specifically in early germ cells via the *nos*:Gal4 driver (Van Doren et al., 1998) (*nos* > how^{RNAi}) and examined adult testes for any spermatogenic defects. Analysis of adult testes from newly eclosed flies with the germ cell marker Vasa and the hub marker Fasciclin III (FasIII) revealed germline depletion and GSC loss in 37% (n = 70) of *nos* > how^{RNAi} testes whereas 27% had lost some GSCs (Figure 2D). The germ cell loss phenotype observed in most of the *nos* > how^{RNAi} testes was more severe than the hypomophic allelic combination in that the few germ cells that were present did not appear in cysts of more than two cells (Figure 2D). In addition, *nos* > how^{RNAi} testes displayed an increase in the number of cells expressing FasIII (Figure 2D) and had additional differentiated cyst cell nuclei near the hub (Figures 2E and 2F). HOW levels in hub cells are not altered in these testes, so the enlarged hub is likely to be a consequence of germ cell loss, which has previously been shown to disrupt cyst cell differentiation in the *Drosophila* testis, with younger cyst cells reentering the mitotic cycle and transdifferentiating to hub-like cells expressing FasIII (Gönczy and DiNardo, 1996).

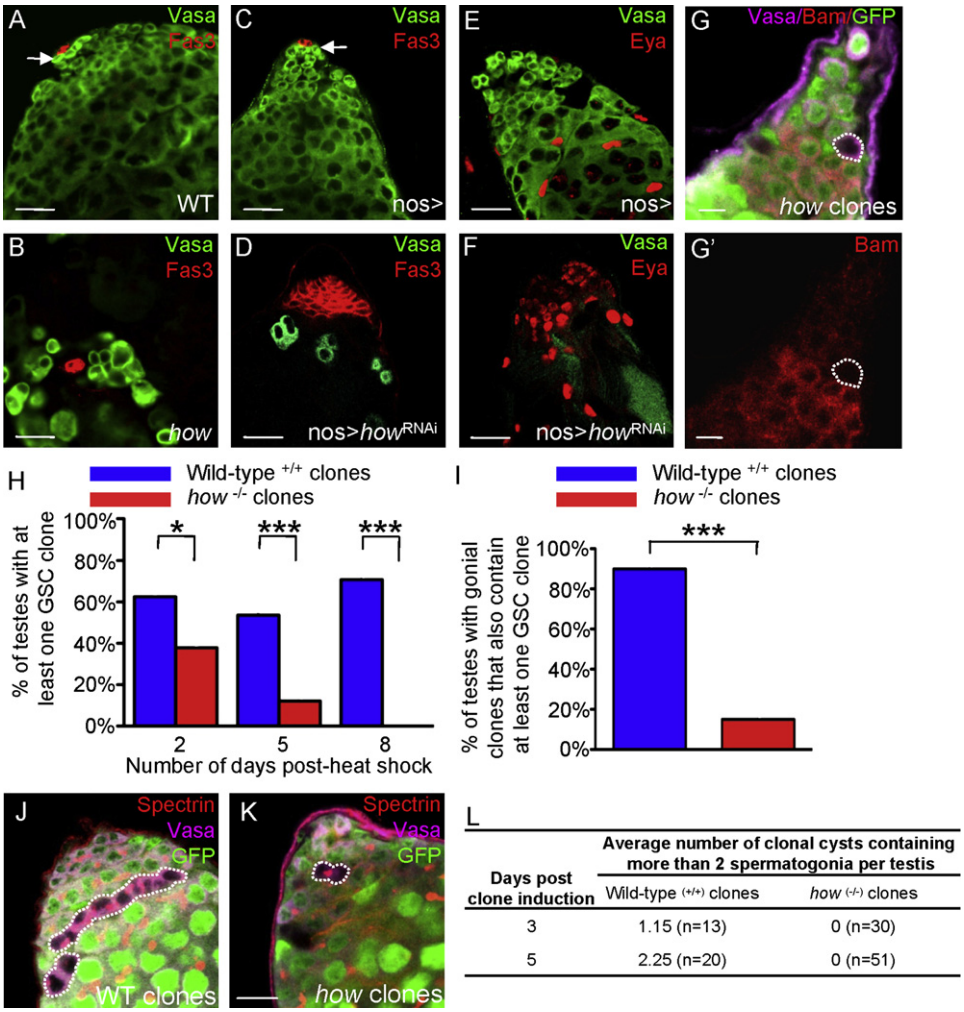
Additional experiments with a second *UAS:*how^{RNAi} construct targeting a different, but partially overlapping, region of the *how* mRNA supported the requirement for HOW in the early germline, because germ cell loss, hub expansion, and cyst cell overproliferation were also observed upon expression of this second construct with *nos*:Gal4 (data not shown). *nos*:Gal4 and *UAS:*how^{RNAi} strains in isolation did not show any defects (Figure S2).

To test for an intrinsic requirement for HOW in GSC division, maintenance, and/or survival, homozygous mutant clones for the strong LOF allele $how^{stru\text{-}3R\text{-}3}$ (Prout et al., 1997) were generated. Two days after clone induction, control GSC clones were observed in 63% of testes (n = 40), whereas homozygous *how* GSC clones were present at lower rates (38% observed, n = 37). Although control GSC clones were maintained after clone induction (5 days 54%, n = 41; 8 days 71%, n = 24), *how* GSC clones were rapidly lost (5 days 12%, n = 43), and at 8 days no GSC clones were observed (n = 21) (Figure 2H). These experiments reveal an intrinsic role for HOW in the division or maintenance of GSC identity.

Gonialblasts undergo four rounds of mitosis over approximately a 2 day period at 25°C before differentiating into spermatocytes (Siddall et al., 2006). Hence, 4 days after clone induction, any one- or two-cell spermatogonial clones observed are assumed to be derived from a clonal GSC, because any clones that were initiated in post-GSC spermatogonia would normally have progressed to the spermatocyte stage. At 4 days after clone induction, 90% of testes that contained WT spermatogonial clones also had at least one GSC clone, which is as expected because the GSC must both self-renew and differentiate (Figure 2I). However, in testes containing *how* mutant clones, only 18% still possessed a progenitor GSC clone after 4 days, indicating that GSCs were more sensitive to the loss of HOW function and were rapidly lost from the niche. In contrast, spermatogonial clones persisted for a longer period of time. We tested whether *how* mutant GSCs were lost from the male germline niche because of premature differentiation by analyzing expression of the differentiation marker, Bam, in germ cells within the stem cell niche. The absence of Bam in *how* mutant germ cells suggested that GSCs lacking HOW function do not differentiate prematurely into spermatogonia (Figures 2G and 2G′).

Germ Cells Lacking *how* Function Fail to Progress Past the 2-Cell Spermatogonial Stage

Five days after clone induction, wild-type clones were regularly observed at all spermatogonial stages (1, 2, 4, 8, and 16 cell cysts) (n = 20; Figures 2J and 2L); however, cysts containing



L

Days post clone induction	Average number of clonal cysts containing more than 2 spermatogonia per testis	
	Wild-type (+/+) clones	*how* (-/-) clones
3	1.15 (n=13)	0 (n=30)
5	2.25 (n=20)	0 (n=51)

Figure 2. HOW Is Required in GSCs for Their Maintenance and for Spermatogonial Proliferation

(A–F) The apex of WT third instar (A) and adult (C) testes contain high levels of Vasa-positive cells (germ cells, green), including GSCs (white arrow), which surround the hub (red). In third instar *how*$^{r17/r4}$ (B) and adult testes derived from germline-specific *how* knockdown (D), germ cells, including GSCs, are lost and the hub is expanded (red). HOW germline knockdown (F) results in more Eya-positive late cyst cells (red) closer to the hub than in WT (E), suggesting loss of early germ cells.

(G) A GFP negatively marked *how* clonal spermatogonium (dotted line) derived from a *how* GSC clone (4 days after clone induction) does not express Bam (red), indicating that GSCs are not lost via differentiation (G′).

(H) Comparison of WT and *how* GSC clone maintenance over time at 2, 5, and 8 days after clone induction indicates that GSCs lacking HOW are lost from the niche. *$p < 0.05$, ***$p < 0.01$.

(I) 90% of testes containing WT spermatogonial clones 4 days after induction also contain a GSC clone compared to only 17.7% of *how* clones, indicating that spermatogonia derived from *how* GSCs lose their progenitor GSC over time. χ^2 tests revealed the difference to be significant, ***$p < 0.01$.

(J and K) Comparison of testes containing WT (J, white line) and *how* (K, white line) germ cell clones. Five days after induction, WT clones derived from WT GSCs have reached the spermatocyte stage (J). Spermatogonia derived from *how* GSCs fail to progress past the 2-cell stage, as seen by the single fusome (red) connecting two germ cells (K).

(L) Cysts containing more than two *how* spermatogonia at either 3 or 5 days after induction were not observed.

Scale bars represent 20 μm for (A)–(F), (J), and (K); 5 μm for (G), (G′). See also Figure S2.

more than two *how* spermatogonia were never observed (n = 51; Figures 2K and 2L). Additionally, *how* germ cells do not express the Bam differentiation marker (Figure 2G′), which is first detectable at the 4-cell stage, confirming that germ cells lacking HOW function fail to reach this stage. Further inspection revealed that the morphology of the *how* 2-cell cysts were abnormal; in particular *how* mutant cells were larger than surrounding WT spermatogonia of the equivalent stage (i.e., the 2-cell cysts; Figure 3B).

Germ Cells Lacking *how* Function Display Increased Cell Size

To investigate the size differential between WT and *how* spermatogonia, we generated *how* clones and compared these

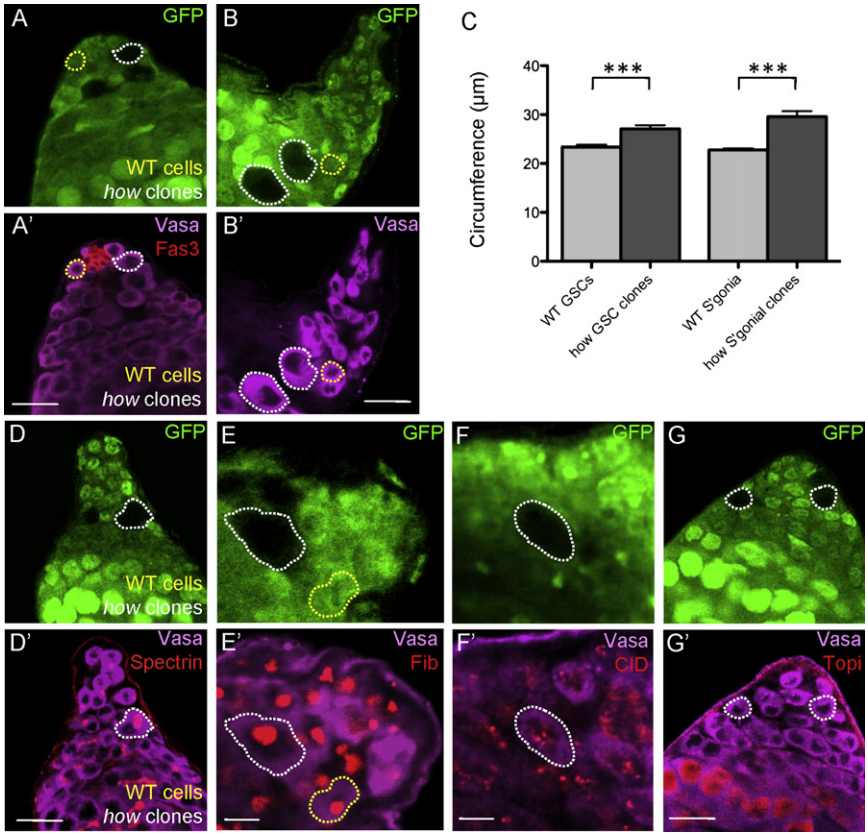


Figure 3. Germ Cells Lacking *how* Function Display Growth Defects

(A, B, D–G) Testes containing *how* germ cell clones (GFP negative, white line) 4 days after induction.

(A) *how* GSC clones are larger than WT GSCs (yellow line).

(B) *how* spermatogonia are larger than adjacent WT spermatogonia (yellow line).

(C) Comparison of germ cell circumferences.

(D) Fusomes (red) connecting *how* spermatogonia were larger than fusomes connecting WT cells.

(E) Nucleoli (red) from *how* spermatogonia are larger than nucleoli from adjacent WT cells (yellow line).

(F) Anti-CID staining shows that *how* spermatogonia contain the correct number of centromeres (red).

(G) *how* spermatogonia do not express the spermatocyte marker, Topi (red), suggesting that they have not initiated differentiation.

Scale bars represent 20 μm for (A), (B), (D), and (G); 5 μm for (E) and (F). Error bars indicate standard error of the mean. See also Figure S3.

directly to neighboring WT cysts. Clones were identified via lack of the GFP marker. They also did not label with anti-HOW (Figure S3A′). We observed that both *how* GSCs and spermatogonia were larger in size than their WT counterparts (Figures 3A and 3B). The mean circumference of a WT GSC was 23.39 μm (n = 48), compared to 27.08 μm for *how* GSC clones (n = 26, $p < 0.0001$; Figure 3C). This size difference was more pronounced in 4-day-old spermatogonia derived from *how* GSCs, which have a mean circumference of 29.57 μm (n = 58) at the 1- or 2-cell stage (the only stages *how* spermatogonia are present) compared to WT spermatogonia that were 22.77 μm, $p < 0.0001$ (n = 44). Furthermore, although *how* spermatogonial clones near the hub were closer in size to WT spermatogonia, the size differential was greater between control cells and *how* spermatogonial clones further from the hub, with more distant cells measuring up to 52.78 μm, more than double the mean WT circumference. Consistent with the clonal analysis, larger germline (Vasa-positive) cells were also observed in *nos* > how^{RNAi} testes, with one observed with a circumference of 80.5 μm (Figure S2).

To confirm the observed cell size increase, we examined intracellular components in WT and *how* spermatogonia. Fusomes that connected *how* sibling cells were observed to be larger than comparable WT cells (Figures 3D and 3D′). In order to determine whether the larger cell size was associated with increased cell growth in the *how* mutant cells, we used Fibrillarin to mark nucleolar size as a measure of ribosome biogenesis, which is essential and rate limiting for growth (Grewal et al., 2005). Consistent with the larger cell size, *how* mutant spermatogonia contained larger nucleoli than their WT counterparts (Figures 3E and 3E′). To test the possibility that the increased cell size was due to tetraploidy resulting from defective cytokinesis, the CID antibody was used to detect centromeres; however, *how* spermatogonia were found to contain the correct number of centromeres (Figures 3F and 3F′). Very few of these cells were observed to incorporate BrdU, also indicating that they were not endoreplicating (Figure 4A). An alternate explanation for the increase in cell size could be that *how* spermatogonia have prematurely differentiated; however, our experiments showed that these mutant cells did not express the spermatocyte markers Topi (Figures 3G and 3G′) and Comr (data not shown), suggesting that premature spermatocyte differentiation was unlikely. *how* mutant cells away from the hub were observed to express the stem cell marker *esg-lacZ*; however, the β-galactosidase enzyme can show some perdurance through several cell divisions, as we observed for WT cells at the same stage (Figures S3B and S3B′). Although *how* cells were connected by fusomes (Figure 3D), indicating that they were spermatogonia, some pairs of cells had fusomes that more resembled the dot-like spectrosomes characteristic of GSCs and gonialblasts (Figures S4G″). It is therefore possible that GSCs lacking HOW migrate away from the stem cell niche, which would explain the GSCs loss in the mutant (Figure 2H).

The Cell Cycle Is Stalled in the G2 Growth Phase in *how* Mutant Germ Cells

We next examined several phase-specific cell cycle markers in WT and *how* 1- or 2-cell spermatogonial clones (4 days after induction). In all testes examined, it was extremely rare to detect *how* LOF spermatogonia-expressing Cyclin B (CycB) (4%,

A

Marker	*how* clones counted (WT clones counted)	*how* clones expressing marker (WT clones expressing marker)	% (WT %)
Cyclin B	57	2	4%
	(43)	(14)	(33%)
Cyclin A	107	95	89%
	(29)	(17)	(59%)
PH3	118	4	3%
	(53)	(3)	(6%)
Dacapo	97	6	6%
	(26)	(14)	(54%)
Geminin	24	24	100%
	(14)	(14)	(100%)
BrdU	48	4	9%
	(95)	(29)	(31%)

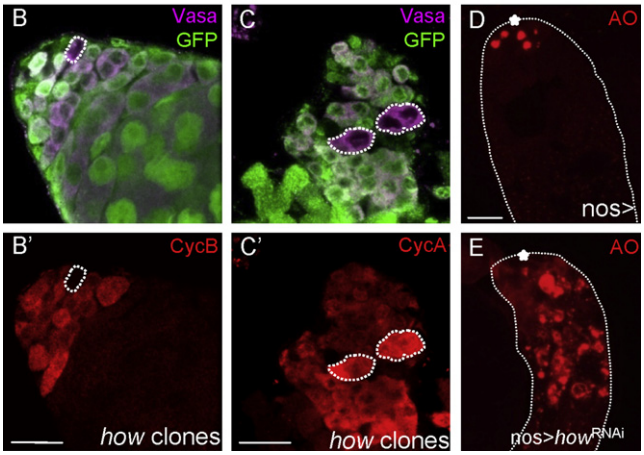


Figure 4. *how* Spermatogonia Are Delayed in G2 Phase

(A) The number of *how* spermatogonia positive for CycB is decreased compared to WT clones 4 days after induction. *how* clones display an accumulation of CycA and Geminin but lack Dacapo, BrdU labeling, and PH3, suggesting that *how* germ cells are delayed prior to prophase in G2 because of lack of CycB.

(B and C) *how* spermatogonial clones (GFP negative, white line) 4 days after induction.

(B) *how* clones display reduced CycB levels (red).

(C) *how* clones are largely CycA positive (red) with higher levels of expression than wild-type CycA-positive spermatogonia.

(D and E) Germ cells lacking *how* function undergo apoptosis as shown by the increased number of acridine orange-positive cells (red) around the hub (*) observed in nos > how^{RNAi} testes (E) compared to WT (D).

Scale bars represent 20 μm for (B) and (C); 100 μm for (D) and (E). See also Figure S4.

n = 57), which normally peaks during the G2 phase of the cell cycle and is degraded at the metaphase-anaphase transition (Sigrist et al., 1995). In contrast, 33% of WT clonal spermatogonia were CycB positive (n = 43; Figures 4A, 4B, and 4B′). To determine whether *how* mutant cells were delayed in G2 because of reduced levels of CycB, or whether cells were arrested in another phase of the cell cycle, *how* mutant germ cells were stained for the other cyclin that peaks in G2 of the cell cycle, Cyclin A (CycA). In contrast to the CycB analysis, 89% of *how* spermatogonia express CycA (n = 107; Figure 4A). Furthermore, the levels of CycA expression were consistently much higher in *how* mutant cells than in WT CycA-positive cells, suggesting that these cells are mostly in preprophase G2 (Figures 4C and 4C′). CycA has also been suggested to coordinate the synchronized divisions of germ cells via its association with the fusome (Lilly et al., 2000). We observed no disruption to the CycA-fusome interaction in *how* germ cells (Figures S4G–S4G″).

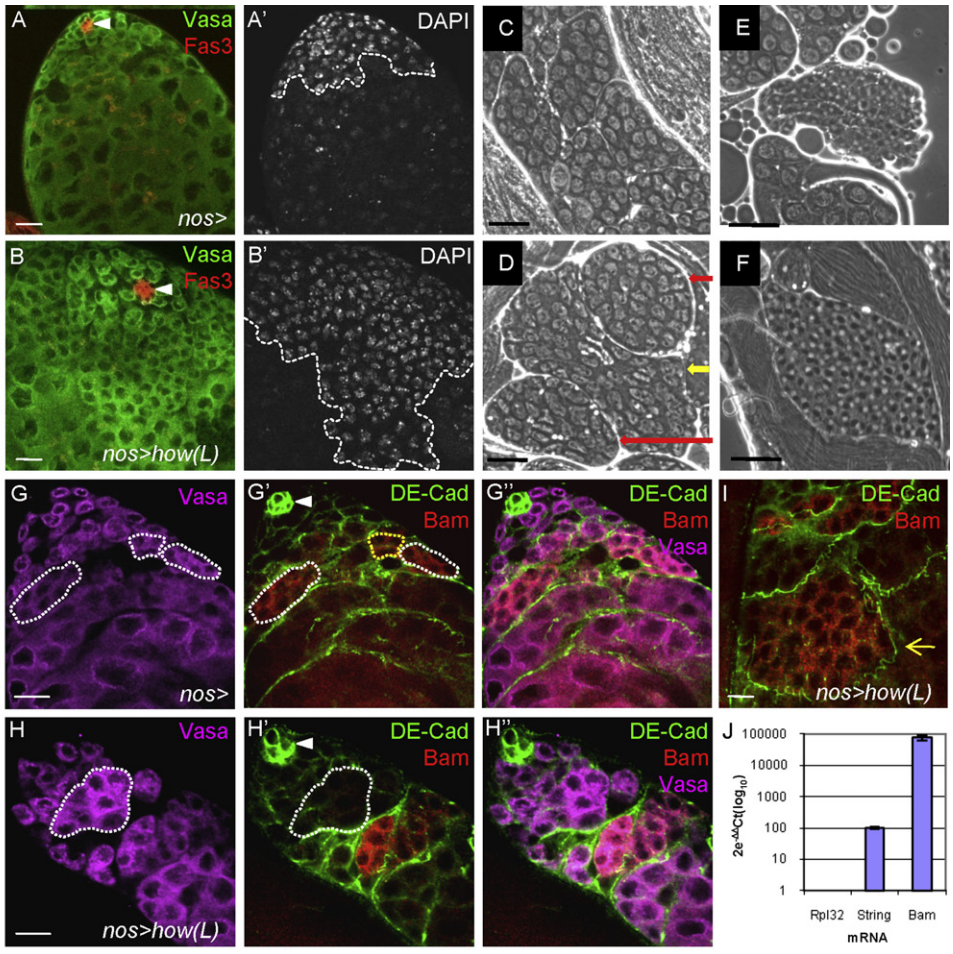
Phospho-Histone H3 (PH3) labels mitotic cells from prophase through to telophase (Su et al., 1998). Consistent with a failure to enter prophase, very few *how* spermatogonia were PH3 positive (Figure 4A). These cells all expressed Geminin (normally present at high levels in G2) (Quinn et al., 2001) and were negative for the G1 regulator Dacapo (Hatfield et al., 2005), again indicating that *how* mutant cells were in G2 (Figure 4A). We also examined the number of cells in the S phase of the cell cycle by counting the number that incorporated bromodeoxyuridine (BrdU) after a pulse label. Normally, 31% of WT clonal spermatogonia incorporated BrdU (n = 95), compared to only 9% of *how* spermatogonia (n = 48; Figure 4A). Together these results are consistent with germ cells lacking *how* function delaying in G2 phase of the cell cycle because of a lack of CycB.

how mutant germ cells are ultimately lost from the testis; therefore, if they do not differentiate they presumably die some time after the G2 delay. We examined whether *how* spermatogonial cells underwent cell death by comparing acridine orange (AO) staining levels and distribution in WT testes and *nos* > how^{RNAi} mutants. As described previously, many mutant testes were agametic, but in testes where germ cells were present, we observed significantly more AO-positive staining cells in *how* mutant testes (Figure 4E; Figure S4H) compared to WT testes (Figure 4D). Taken together, our results indicate that germ cells lacking *how* function fail to progress past the 2-cell stage because of a delay in the G2 phase of the cell cycle and are eliminated from the germline via apoptosis.

To determine whether HOW function in the germline was entirely mediated via CycB, we asked whether overexpression of CycB could rescue the germ cell loss phenotype observed in *nos* > how^{RNAi} testes by coexpressing *UAS:cycB* with *nos*:Gal4 (*nos* > *cycB*;how^{RNAi}). As mentioned above, 37% of adult *nos* > how^{RNAi} testes had no GSCs, 27% showed reduced spermatogonia with spermatocytes near the hub, and 23% had normal germ cell architecture (Figures S4B and S4F). In *nos* > *cycB*; how^{RNAi} testes, just 7% had no GSCs and 15% had reduced spermatogonia with spermatocytes near the hub, whereas 75% had the normal germ cell architecture (n = 27; Figures S4C and S4F). These results suggest that the majority of HOW function in the early germline is mediated via CycB. Conversely, removing one copy of WT *cycB* in *nos* > how^{RNAi} flies enhanced the germ cell loss phenotype with 100% of testes appearing agametic (n = 14; Figures S4D and S4F). *nos:*Gal4 (9.9 ± 1.7, n = 13), $cycB^{+/-}$ (11.4 ± 1.9, n = 16), and *nos* > *cycB* (10.2 ± 0.8, n = 17) all had normal numbers of GSCs.

HOW(L) Controls the Transition from Mitotic Spermatogonia to Differentiating Spermatocytes

In order to determine whether high levels of HOW could promote extra germ cell divisions, we overexpressed a *UAS:how(L)* construct in the germline via the *nos*:Gal4 driver (*nos* > *how(L)*). Initial observations indicated that *nos* > *how(L)* testes were larger in size than WT, with the most notable difference being an expansion of the apex of the testis (Figure 5). Vasa and DAPI staining revealed an increase in the number of early germ cells in *nos* > *how(L)* testes compared to WT as shown by the increase in small Vasa-expressing cells with intense DAPI staining at the testis



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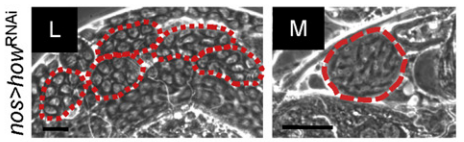
Genotype	Bam Expression			
	4-cell +	4-cell -	8-cell +	8-cell -
nos>+	26	0	23	0
nos>how(L)	10	5	23	1



N

Genotype	Spermatocytes within cyst				
	8	16	32	64	Total
nos > how(L); +/+*	0	164	1	0	165
bam$^{\Delta 86}$/+	0	94 (71%)	36 (27%)	3 (2%)	133
nos >how(L); bam$^{\Delta 86}$/+*	0	68 (30%)	144 (65%)	11 (5%)	223
bam$^{\Delta 86}$/how^{stru}	0	102 (99%)	1 (1%)	0	103
how^{stru}/+	10 (9%)	97 (91%)	0	0	107

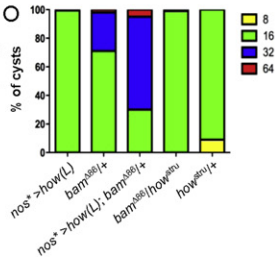


Figure 5. Ectopic Expression of HOW(L) in the Germline Results in Extra Rounds of Mitosis by Repressing Bam

Comparison of WT (A, C, E, G) to *nos > how(L)* (B, D, F, H, I).

(A) Spermatogonia are Vasa positive (green) and the nuclei stain intensely with DAPI (A′, white line).

(B) *nos > how(L)* results in excess rounds of spermatogonial mitoses as shown by the increased number of Vasa-positive cells surrounding the hub (green) (B) and intensely DAPI-positive cells (B′, encapsulated by white line).

(C–F) Phase-contrast microscopy of a WT testis shows cysts containing 16 spermatocytes (C) and 64 early spermatids (E). In *nos > how(L)* testes, cysts containing 32 (red arrow) or 64 (yellow arrow) spermatocytes (D) and 128 early spermatids (F) are observed.

(G) In WT testes, Bam (red) can be detected in germ cells (magenta) at the 4-cell, 8-cell, and 16-cell spermatogonial stage. Hub and cyst cells (green). 4-cell cyst (outlined in yellow) and 8-cell cysts (outlined in white) in (G′).

(H) In *nos > how(L)*, Bam expression is not detected in some 4-cell and 8-cell (white line) spermatogonial cysts.

(I) *nos > how(L)* testis shows Bam (red) in a 32-cell spermatogonial cyst (yellow arrow).

(J) Enrichment of *string* and *bam* mRNA, relative to *RpL32*, after HOW immunoprecipitation. Error bars indicate standard error of the mean.

apex (Figures 5A′ and 5B′). This suggested that the overexpression of HOW(L) in the germline caused a hyperproliferation of spermatogonial cells.

Excess early germ cells can be produced by an expansion of the stem cell population and subsequent cell division, or by a delay in the normal program of differentiation resulting in extra rounds of spermatogonial mitosis. To determine whether this hyperproliferation was due to spermatogonia proceeding through extra rounds of mitosis, we dissected adult testes and flattened live preparations under a coverslip for phase-contrast microscopy in order to count the number of spermatocytes within individual cysts. In WT testes, the maximum number of spermatogonia within a cyst is 16, which differentiate into 16 spermatocytes (Figure 5C). In *nos > how(L)* testes, cysts of 32 and 64 spermatocytes were frequently observed (Figure 5D), which suggests that overexpression of HOW(L) in the germline results in spermatogonia undergoing either one or two extra rounds of mitoses. This failure to exit the mitotic divisions was not permanent, as shown by the fact that germ cell tumors were not apparent, and spermatogonial cysts with increased numbers could differentiate into spermatocytes. We never observed cysts containing more than 64 spermatocytes, although it should be noted that enlarged cysts are very fragile and may not survive the live cell observation procedure. Cysts containing 32 spermatocytes were also able to undergo meiosis correctly; cysts of 128 early spermatids were observed (Figure 5F), which is double the number seen in WT (Figure 5E).

The HOW(L) overexpression phenotype is similar to that observed in *bam* heterozygotes. Bam induces the switch from mitotic proliferation of TA spermatogonia to spermatocytes when it reaches a critical threshold (Insco et al., 2009). *bam* mRNA was a likely target of translational repression by HOW as indicated by the fact that the expression pattern of HOW is complementary to Bam in the early germline (Figures 1B–1E). We immunoprecipitated HOW bound to its target mRNAs from embryos via anti-HOW and reverse transcribed the mRNA before amplifying potential targets via quantitative real-time PCR (qRT-PCR). We used embryonic extracts because HOW has known targets in embryos (Nabel-Rosen et al., 2005) and thus *stg* mRNA could be used as a positive control. Additionally, *bam* mRNA is found at high levels in early embryos. We normalized the qRT-PCR data against *RpL32* mRNA (or *rp49*), a ubiquitous component of ribonuclear complexes that does not contain a consensus HOW binding site. As predicted, immunoprecipitation with anti-HOW resulted in enrichment for *stg* (93-fold). However, enrichment for *bam* via anti-HOW was 842-fold higher than for *stg* (78,300-fold compared with *RpL32*) (Figure 5J). These data suggest that HOW is likely to regulate Bam by forming a complex with the *bam* mRNA to repress translation. We therefore examined the effect of ectopic HOW(L) on Bam expression in the testis. All 4-cell (n = 26) and 8-cell (n = 23) cysts of spermatogonia were observed to label with anti-Bam in control testes (Figures 5G–5G″ and 5K). In *nos > how(L)* testes, 50% of 4-cell cysts (n = 10) and 4% of 8-cell cysts (n = 23) did not express Bam, indicating that ectopic HOW(L) caused a delay in the expression of Bam.

Extra gonial divisions have been reported in $bam^{\Delta 86}$/+ males (Insco et al., 2009). In our study, 27% of spermatocyte cysts contained 32 cells and 2% contained 64 cells in $bam^{\Delta 86}$/+ testes (Figures 5N and 5O). We employed a weak *nos:Gal4* driver (*nos**) that only rarely resulted in extra mitotic divisions (<1%) when combined with *UAS:how(L)* (Figures 5N and 5O). In *nos* > how(L);*$bam^{\Delta 86}$/+ testes, 65% of cysts contained 32 spermatocytes indicating a significant enhancement of the $bam^{\Delta 86}$/+ phenotype (Figures 5N and 5O).

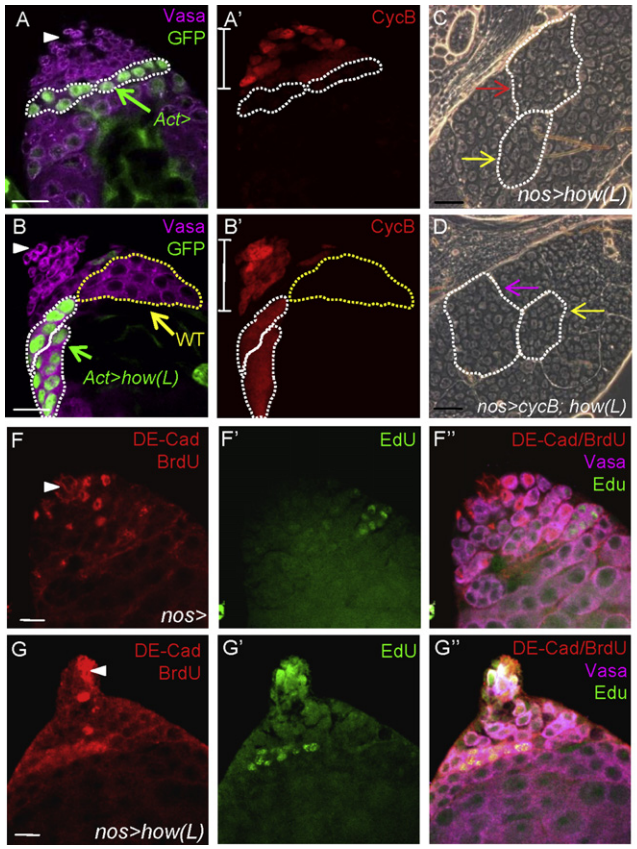
Although ectopic HOW(L) can delay differentiation of spermatogonia, these data did not indicate whether endogenous HOW regulates the number of TA divisions. Halving the dose of HOW resulted in generation of 8-cell cysts of spermatocytes as well as suppressing the extra divisions present in $bam^{\Delta 86}$/+ males (Figures 5N and 5O). In addition, some of the spermatogonial cysts in *nos >* how^{RNAi} transgenic testes did not arrest at the 2-cell stage but differentiated early into 8-cell spermatocyte cysts (Figure 5L). These cysts could complete meiosis and differentiate into cysts containing half of the normal complement of spermatids (Figure 5M).

Together these data demonstrate that HOW regulates accumulation of Bam and determines the number of TA mitoses prior to differentiation. We also overexpressed HOW(S) in early germ cells but did not observe any phenotype (Figure S5C), suggesting that either HOW(S) cannot compete for targets with HOW(L) or that it plays little role in the germline. Ectopic HOW(S) shows a predominantly cytoplasmic localization (Figure S5C) distinct from the nuclear localization of ectopic HOW(L) (Figure S5B) or endogenous HOW (Figure S5A). Both forms of *how* transcript can be detected in the testis by RT-PCR (Figure S5H) and, surprisingly, ectopic expression of either form can rescue the phenotype exhibited by the *how* hypomorphic allelic combination (Figures S5D–S5G). This may indicate that the model of opposing functions of HOW(L) and HOW(S) may not represent the mechanism of HOW function in all tissues.

HOW(L) Also Regulates Levels of CycB and the Rate of Spermatogonial Cell Cycles

CycB is normally downregulated at the end of the mitotic divisions and is not detectable in early spermatocytes (White-Cooper et al., 1998). Expression of CycB is reinitiated as cells enter meiosis I. Because loss of HOW led to reduced CycB expression, we also hypothesized that ectopic HOW(L) may lead to maintenance of CycB levels beyond the stage where it

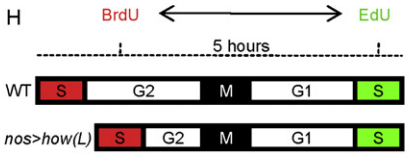
(K) In *nos > how(L)* testes, Bam accumulation is delayed in 4-cell cysts compared to WT ($p = 0.001$).
(L and M) In *nos >* how^{RNAi} testes, spermatogonia that do not stall at the 2-cell stage differentiate prematurely as seen by the presence of 8-cell spermatocyte cysts (L) and 32-cell spermatid cysts (M).
(N and O) *bam* and *how* genetically interact in the male germline. A weak *nos*:Gal4 driver (*nos**) has little effect in combination with *UAS:how(L)*; however, *nos* > how(L)* enhances the $bam^{\Delta 86}$/+ phenotype, as shown by the fact that a larger proportion of cysts contain 32 cells compared to $bam^{\Delta 86}$/+ ($p < 0.0001$). Conversely, removal of one copy of *how* suppresses the $bam^{\Delta 86}$/+ phenotype, as shown by the fact that 99% spermatogonia differentiate in cysts of 16 cells ($p < 0.0001$). 9% of how^{stru}/+ testes contain cysts of 8-cell spermatocytes. Hub marked with white arrowhead.
Scale bars represent 10 μm for (A), (B), (G)–(I); 50 μm for (C)–(F), (L), (M). See also Figure S5.



E

Genotype	Spermatocytes within cyst: 16	32+	Total Cysts
nos>how(L)	20	83	105
nos>cycB; how(L)	3	104	107

H



I

	Edu Positive Cysts of Spermatogonia: 4 cell cyst		8 cell cyst		16+ cell cyst	
	BrdU		BrdU		BrdU	
Genotype	+	-	+	-	+	-
nos>WT	0	17	0	24	0	16
nos>how(L)	3	4	5	14	8	12

Figure 6. HOW(L) Regulates the Timing of Spermatogonial Divisions

(A) CycB is normally observed in GSCs and spermatogonia up to the 8-cell stage (A′). When HOW(L) is overexpressed in GFP positively marked spermatocytes (green cells in B), CycB (B′) can be detected (white line). WT spermatocytes (yellow line) do not express CycB similar to control clones (white line in A).

(C and D) Phase-contrast microscopy showing cysts of spermatocytes (white line).

(C and E) *nos > how(L)* results in extra rounds of spermatogonial mitosis. Cyst containing 16 (yellow arrow) and 32 (red arrow) cells.

(D and E) *nos > cycB,how(L)* results in a higher proportion of cells undergoing extra rounds of mitosis ($p = 0.0001$); 32 (yellow arrow) and 64 (magenta arrow) cell cysts.

(F–I) Analysis of the rate of the spermatogonial cell cycle in WT cells and *nos > how(L)* testes.

(H) Representation of experimental paradigm.

(F–F″) Pulse labeling of BrdU (red) and EdU (green) at 5 hr intervals shows no WT spermatogonia incorporate both S phase markers.

(G and I) In *nos > how(L)* testes, some spermatogonial cells incorporate both S phase markers, indicating that they have completed one cycle in under 5 hr. Hub and cyst cells marked (red). Hub marked with white arrowhead.

Scale bars represent 20 μm for (A) and (B); 10 μm for (F) and (G). See also Figure S6.

is normally downregulated and thereby influence spermatogonial mitoses. We examined this possibility by using *Actin > CD2 > GAL4* to generate "flip-out" clones overexpressing HOW(L). As expected in WT testes, CycB was detected in the GSCs and spermatogonia up to the 8-cell stage (Figure 6A′). Induction of WT GFP-positive control clones in spermatocytes did not affect CycB levels (Figures 6A and 6A′). However, when HOW(L) was overexpressed in germ cells, CycB expression was observed in early spermatocytes (Figures 6B and 6B′), suggesting that overexpression of HOW(L) in late-stage germ cells prevents the reduction of CycB.

Ectopic expression of CycB does not phenocopy the *nos > how(L)* phenotype (Figure S6B), indicating that increased levels of CycB do not result in excess mitoses; however, ectopic CycB does enhance the number of extra spermatogonial mitoses observed in *nos > how(L)* testes (Figure 6E). This made us question whether ectopic HOW(L) affected the spermatogonial cell cycle. Insco et al. (2009) measured the relative rates of spermatogonial cycles by labeling testes with BrdU and then EdU after an interval. Cells that were not double labeled were deemed to have taken longer than the interval to go through subsequent S phases. We modified this protocol because we conducted a comparison between *nos >* and *nos > how(L)* testes at 29°C and found spermatogonial cell cycles to be more rapid at this increased temperature. No control spermatogonia were found to be double labeled under this regime (Figures 6F–6F″ and 6I), but a proportion of 4-cell, 8-cell, and 16+-cell spermatogonial cysts labeled with both nucleotide analogs, indicating that cell cycle time was shortened in spermatogonia that express ectopic HOW(L) (Figures 6G–6G″ and 6I).

HOW was also observed to be expressed in hub cells and maturing cyst cells but not SSCs (Figures 1B and 1E), so we

tested the requirement for HOW function in these tissues. *Upd:Gal4* drives expression in hub cells of the testis (Kawase et al., 2004) and *Upd* > *how*^{RNAi} resulted in loss of the hub and subsequent loss of early germ cells (Figures S6B–S6B″). *c587:Gal4* drives expression in SSCs and cyst cells (Kawase et al., 2004). *c587* > *how*^{RNAi} did not exhibit any stem cell niche defects; however, the germ cells associated with maturing cyst cells were distinctly abnormal (Figures S1D–S1D″). We observed loss of cysts of spermatocytes and overproliferation of germ cell cysts, the latter phenotype being associated with cyst cell dysfunction (Sarkar et al., 2007). MARCM-generated *how*$^{-/-}$ SSC clones were observed at similar levels (18/54) to control clones (11/41) in 7-day-old testes. These data indicate that loss of *how* does not affect SSC regeneration or production of cyst cells but HOW function is required in postmitotic cyst cells. HOW is therefore playing different roles in the somatic lineages compared with the germline.

DISCUSSION

Regeneration within a stem cell niche requires that (1) the stem cell population is capable of cycling, (2) the TA daughters of a stem cell division can rapidly expand the undifferentiated cell population and, (3) the TA cells leave the cell cycle in order to differentiate. Here we show that in the *Drosophila* testis, the RNA-binding protein HOW is required to maintain GSCs and spermatogonial cell divisions. Loss of HOW results in reduced levels of CycB, with an associated G2 delay, which potentially results in increased apoptosis. Furthermore, HOW overexpression, which causes perdurance of CycB beyond the spermatogonial stage and delays expression of Bam, is associated with additional spermatogonial mitoses after the normal four divisions. Therefore, this study has identified HOW as a component of the regulatory machinery that maintains GSCs and modulates the early steps of cyst formation.

HOW Is Required for Early Spermatogonial Mitoses

Analysis of *how*$^{-/-}$ GSC clones revealed an intrinsic role for HOW in maintaining GSCs and for permitting mitotic amplification of spermatogonial cells. The decreased levels of CycB and G2 arrest in *how* mutant germ cells is associated with increased cell size and is followed by elimination via apoptosis. The observation that loss of *how* results in two separate germ cell phenotypes (i.e., loss of GSCs and arrest at the 2-cell spermatogonial stage) was initially surprising. However, this may simply reflect the mechanism of clone generation. Flp-mediated mitotic exchange that occurs in an asymmetric GSC division can produce a *how*$^{-/-}$ GSC and a *how*$^{+/+}$ gonialblast or the reciprocal cell types. We did not observe arrested gonialblasts but 2-cell spermatogonia, suggesting that mutant cells can progress through a single division before arresting in G2. This could indicate that existing HOW protein might be stable through a single cell cycle after generation of *how*$^{-/-}$ clones. We also observed that some arrested 2-cell cysts contained fusomes that more resembled dot-like spectrosomes (Figure S4G″), indicating that GSCs/gonialblasts may contribute to GSC loss by leaving the stem cell niche.

HOW protein expression was not observed in SSCs and loss of HOW function in these cells did not affect their maintenance or ability to differentiate into cyst cells (Figure S1).

HOW(L) has previously been shown to repress gene expression by binding to the 3′UTRs of target mRNAs and inducing their degradation (reviewed in Volk et al., 2008). The finding that CycB is downregulated upon loss of HOW function suggests that HOW is unlikely to directly regulate CycB protein expression, but may repress expression of a negative regulator of CycB. We did detect an interaction between HOW and *cycB* mRNA in our immunoprecipitation experiments but it was at a level 252-fold lower than the HOW:*bam* mRNA interaction, and direct negative regulation of CycB is not consistent with our genetic studies.

Primordial germ cells are known to suppress mitotic activity during their migration into the embryonic gonad by repressing CycB translation mediated via a complex of Nanos and Pumilio (Asaoka-Taguchi et al., 1999). We observed no effects on levels of either of these two translational repressors in *how*$^{-/-}$ cells (data not shown). Ectopic expression of HOW(L) in the germline resulted in perdurance of CycB protein at the time that spermatogonial cells normally degrade CycB and exit the mitotic cell cycle and caused spermatogonia to undergo ectopic mitotic divisions. We never observed this phenotype by directly driving a CycB transgene, and *nos* > *cycB* testes exhibited the wild-type pattern of CycB expression (Figure S6), suggesting that HOW may suppress the mechanism that degrades CycB protein at the cessation of the mitotic cycles. Although our data suggest that the primary mechanism by which HOW regulates transition to the differentiation phase is via regulation of Bam accumulation, we also observed an increase in the rate of spermatogonial cell cycles as a result of HOW(L) overexpression (Figure 6). This would have the effect of allowing extra divisions during the period of Bam accumulation, consistent with the 32- and 64-cell spermatocytes we observed. Although ectopic CycB is not sufficient to increase cell cycle progression, increased CycB enhances the HOW(L) phenotype, suggesting that HOW(L) affects a second factor that is rate limiting for CycB-dependent division.

HOW(L) Regulates the Exit from the Spermatogonial Transit-Amplifying Divisions

The transition from spermatogonial mitotic cycles to differentiated spermatocytes is regulated by levels of Bam. Bam begins to accumulate in 4-cell spermatogonia and is maximal in 8-cell spermatogonia, dropping abruptly after completion of premeiotic S phase in 16-cell cysts. Recent work has demonstrated that spermatogonia must express a threshold level of Bam in order to differentiate (Insco et al., 2009). The complementary expression patterns of HOW and Bam in the early germline first alerted us to the possibility that Bam was a target of HOW repression. Indeed, *bam* mRNA was immunoprecipitated with HOW antibody, and we demonstrated that ectopic HOW(L) resulted in a 1–2 cell delay in the accumulation of Bam protein (Figure 5). This has the effect of allowing an extra 1–2 cell divisions prior to cells reaching critical Bam threshold and will result in production of spermatocyte cysts containing 32 and 64 cells. Lowering HOW levels could suppress the delay to differentiation exhibited by *bam* heterozygotes, also suggesting that HOW regulates Bam levels (Figures 5 and 7).

Experiments with temperature-sensitive *stat* alleles have demonstrated that spermatogonial cells have the capacity to dedifferentiate into GSCs when the stem cell niche becomes depleted. This capacity for dedifferentiation requires a continued

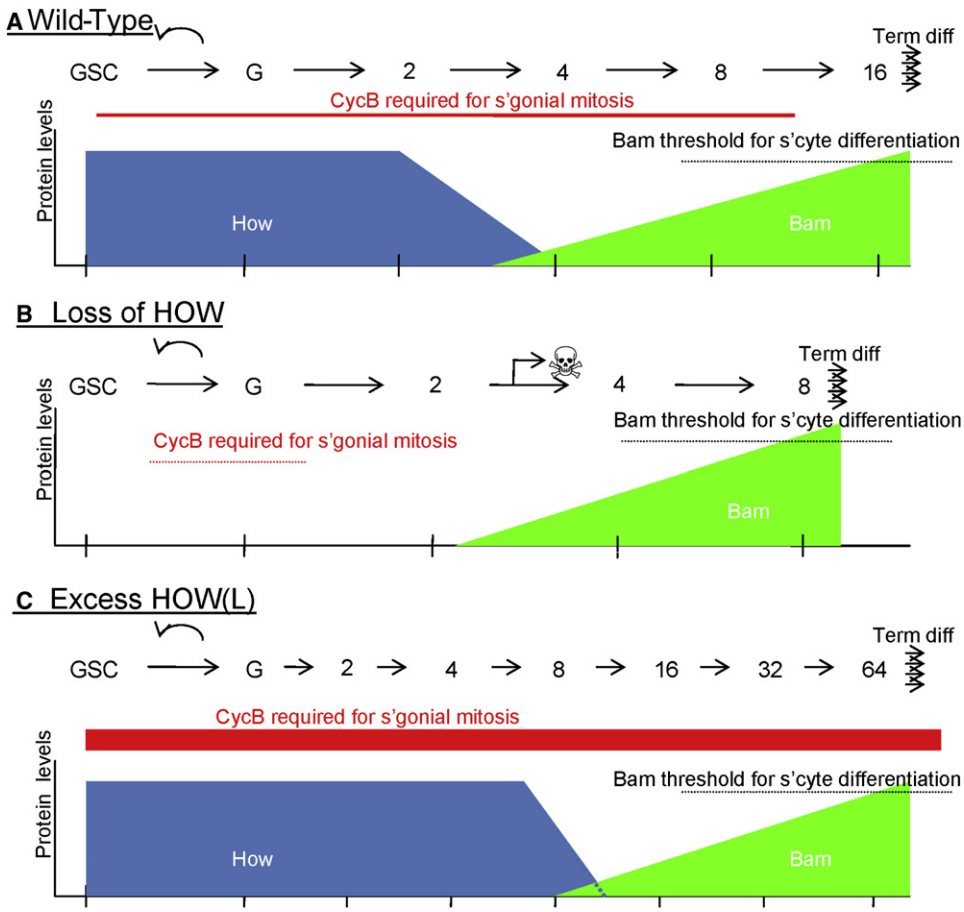


Figure 7. Model of HOW Requirement in Male Germline Cells

(A) WT: HOW expression (blue). Bam (green) levels are first detected at the 4-cell spermatogonial stage and accumulate until a threshold is reached at which point differentiation is induced.

(B) Loss of HOW either results in G2 delay and failure of GSCs or gonialblasts to proceed beyond the 2-cell spermatogonial stage because of the absence of CycB (red), or prematurely differentiate presumably because of earlier Bam accumulation and slower cell cycle.

(C) Overexpression of HOW(L) represses Bam accumulation, causing extra mitoses prior to differentiation. Overexpression of HOW(L) also increases the rate of the spermatogonial cell cycle.

ability for mitotic proliferation because spermatocytes cannot regenerate GSCs (Brawley and Matunis, 2004). A higher number of TA spermatogonia will also provide a greater capacity for regenerating a damaged stem cell niche. Regulators such as HOW may influence not only the mitotic program of germ cells but in turn their ability to regenerate functional stem cells. This study highlights the complex mechanisms that govern cell cycle progression and differentiation within the male GSC niche.

EXPERIMENTAL PROCEDURES

Cytology

Testes were viewed live under phase-contrast microscopy or fixed and immunostained as per Bunt and Hime (2004). Serial confocal sections were taken on either a Biorad MRC1024 or Zeiss LSM510 Confocal Microscope. Cell measurements were conducted with LSM Image Browser software. Testes were incubated in 100 μg/ml BrdU for 30 min in Schneider's medium plus 10% fetal calf serum at 25°C to label S phase. Testes were washed 3× and fixed in 4% paraformaldehyde (in PBS) for 75 min at room temperature prior to washing 3× and incubating with DNaseI (Promega) for 30 min at 37°C prior to immunostaining. BrdU/EdU double labeling was conducted as per Insco et al. (2009) but the interval between labeling was adjusted to 5 hr. Acridine orange staining was performed on adult testes younger than 3 days post-eclosion according to McCall et al. (2004) and analyzed with BB Thermometer v1.4 (c/o BenBritten.com). Statistical analyses were performed with Graphpad Prism and reported as mean ± standard error of the mean. p values were obtained by Student's t test or by χ^2 test for analysis of percent of testes carrying GSC clones.

Generation of Marked Clones

GFP negatively marked homozygous clones were generated with the heat shock-inducible Flp-FRT system. *hs*-FLP/Y; FRT82B *how*^{stru}/FRT82B *Ubi*-GFP or *hs*-FLP/Y; FRT82B/FRT82B *Ubi*-GFP adult males were heat shocked at 37°C for 1 hr and raised at 25°C for the appropriate length of time. Control twinspot clones were counted after immunostaining for Geminin and Dacapo. GFP positively marked HOW(L)-overexpressing clones were generated with the Actin > STOP > GAL4 system by heat shocking adult males at 37°C for 45 min and subsequent incubation at 25°C for 4 days.

MARCM clones were generated as per Leatherman and Dinardo (2008) except that flies were allowed a 2 hr recovery between heat shocks.

Detection of HOW Target mRNAs

Embryos were homogenized by grinding gently in 150 μl polysome lysis buffer containing 0.5% Triton X-100 supplemented with 1 mM Dithiothreitol,10 μl/ml

ProtoCEASE protease inhibitor (G-Biosciences, St. Louis, MO, USA), and 100 units/ml RNasin (Promega Madison, WI, USA). Homogenate was sonicated to disrupt nuclear membranes, lysate centrifuged, and supernatant incubated overnight at 4°C with HOW antibody-coated Protein A Dynabeads magnetic beads prepared according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). After incubation, the bead-Ab-Ag complex was washed in buffer containing protease and RNA inhibitors and resuspended in TES buffer (10 mM TrisHCL [pH 7.5], 1 mM EDTA, 1% SDS) prior to RNA elution and quantification. Quantitative PCR conditions were optimized and target specificity confirmed with cDNA prepared from embryo lysate mRNA. HOW-bound RNA was eluted from beads and collected in DEPC water prior to reverse transcription and analysis by quantitative real-time PCR with an Opticon 2 real-time thermocycler (Bio-Rad Hercules, CA, USA).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at doi:10.1016/j.stem.2010.02.016.

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